

Menstrual blood-derived mesenchymal stem cells provide new insights into the treatment of coronavirus disease 2019 (COVID-19)

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Research

Keywords: coronavirus disease 2019 (COVID-19); mesenchymal stem cell; acute respiratory distress syndrome; pulmonary fibrosis; stem cell therapeutics.

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Abstract

Background: The coronavirus disease 2019 (COVID-19) causing a cluster of respiratory infections in Wuhan, China, is identified in December 2019. The main symptoms are defined as fever, cough, shortness of breath, with early symptom of sputum, acute respiratory distress syndrome (ARDS), and the final lung injury and pulmonary fibrosis. Currently, there is no effective method to cure it. Mesenchymal stem cell (MSC) therapy is an immediate need for treating COVID-19 especially severe patients at present.

Methods: We describe the two confirmed case of COVID-19 severe patients in Hangzhou, China to explore the role of menstrual blood-derived MSC in the treatment of SARS-CoV-2 infection. Furthermore, we mimic disease model of pulmonary fibrosis in mice to assess the role of MSC. Then, a co-culture system to investigate the underlying mechanism between MSC and pulmonary-associated cells by a series of Physiological, biochemical, bioinformatics analysis.

Results: MSC transplantation increases the immune indicators (including lymphocytes) and decreases inflammatory indicators (such as IL-6, IL-10, TNF, and IFN). More importantly, the two patients alleviated symptom and discharged after 3 weeks' treatment with MSC. Additionally, MSCs exhibit an anti-inflammatory role through suppressing some inflammatory factors (RANTES, GM-CSF, MIG-1g, MCP-5, Eotaxin), which is anastomotic to current clinical study using MSC to treat COVID-19.

Conclusions: This is the first report using menstrual blood-derived MSC in treating COVID-19 patients. From our clinical results, we hold one idea that MSCs reduced inflammatory effect to defend cytokine storm. The underlying mechanism is probably that MSCs inhibit epithelia cell apoptosis and reduce the secretion of inflammatory factors to prevent myofibroblasts activity. MSC provides an alternative method for treating COVID-19 particularly some patients with ARDS or subsequent pulmonary fibrosis.

Trial registration: This clinical trial was submitted to and approved by the Ethics Committee of the First Affiliated Hospital, Collage of Medicine, Zhejiang University. MSC administration in patient with COVID-19 was conducted in a single center and open-label clinical trial (ChiCTR2000029606).

Background

The coronavirus disease 2019 (COVID-19) causing a cluster of respiratory infections in Wuhan, Hubei Province, China, was identified in December 2019 [1–3]. It has garnered global attention for that COVID-19 is a highly infectious disease, and has rapidly spread to in many Provinces of China [4–6]. Recently, the COVID-19 infection outbreaks in more and more countries. The number of infected patients has risen rapidly due to a lack of enough awareness, proximity between people, and the human-to-human transmission, as demonstrated in a few family clusters infected by this virus [4, 7]. As of 28 April, 2020, a total of 84,347 reports of confirmed patients, and 4,643 death cases in Chinese mainland have been diagnosed with the SARS-CoV-2 infection from the National Health Commission of China. Currently, epidemiological investigations revealed that many initial patients were exposed to the live seafood and

wildlife market [8]. Thus, the host of infectious source is still supposed to be a kind of wild animals (e.g., bats, pangolin, poultry, and snakes) [9–11].

Acute respiratory distress syndrome (ARDS) and respiratory failure are major lung-associated diseases in COVID-19 patients via the autopsy or biopsy [12]. COVID-19 can cause ARDS and corresponding multi-organ dysfunction with lung inflammatory lesions and structural damage. Hence, a breakthrough in treatment strategy would be critical for treating COVID-19 and especially ARDS-induced severe pneumonia, which is currently no vaccine available for preventing COVID-19 infections. Although COVID-19 patients suffering ARDS at the first stages, however, eventually many of patients develop pulmonary fibrosis. Pulmonary fibrosis is a fatal interstitial lung disease, diagnosed as inflammatory lesions and structural damage [13], most patients are survival less than 5 years [14]. Therefore, how to defense COVID-19 induced ARDS and subsequent induced pulmonary fibrosis will be a key point to thoroughly curing COVID-19 patients. Currently, no specific drugs are found to be effective to clear the viral resistance of COVID-19 and secondary infection induced multiple organ dysfunction in patients is still a serious issue. Therefore, there is an urgent demand to find an effective treatment strategy for COVID-19 infection in humans.

As efforts to controlling ARDS via pharmacological agents have been unsuccessful, mesenchymal stem cell (MSC)-based therapy has been investigated because of MSC's limitless self-renewal and multipotency [15, 16]. Furthermore, MSC-based therapies demonstrated promising effects in experimental acute respiratory distress syndrome (ARDS) via inhibition of alveolar collapse, collagen accumulation and cell apoptosis in lung tissue. Recently, allogeneic MSCs in 9 patients with ARDS, Wilson et al. found that there were no prespecified adverse events including hypoxaemia, cardiac arrhythmia, and ventricular tachycardia [17]. Additionally, our team have proved that MSC therapy is an optional way to treat epidemic Influenza A (H7N9) infection induced ARDS, and further suggesting that MSC transplantation significantly lower the mortality compared with control group [18]. And MSC transplantation has not exert harmful effects in human body in the 5 years' follow up. Currently, menstrual blood-derived MSC is attracting interest due to wide range of source, high proliferation rate, and painless procedure free of ethical issue [19–21].

This study is the first trial to test menstrual blood-derived MSCs in patients with current COVID-19, and we report the effects of MSC transplantation in COVID-19 patient acted as the clinical pilot study, furthermore, we improvement of pulmonary function from COVID-19 infection after MSC transplantation. Furthermore, we utilize bleomycin (BLM) induced pulmonary fibrosis in mice to explore underlying therapeutic effect. Our study will not only contribute to show the function of MSCs in COVID-19 and following complication (especially pulmonary fibrosis) as clinical pilot report, but also suggest that MSCs would be promising tool for treating acute and chronic pneumonia in future clinical use.

Methods

Source and preparation of MSCs

The allogeneic MSCs were obtained from healthy female donors (age 20–45), and notify the volunteers and sign informed consent. At 70–80% confluence of the MSCs, the product was washed, harvested, and cryopreserved. Karyotyping/G-banding was normal by the previous study [22]. The total volume of the MSC infusion was 100 mL regardless of dose. The viability ranged from 90–95%. The surface markers and three-line differentiation of MSCs are conducted as previous study [22].

Treatment for two patients

The Research Ethics Committee of the First Affiliated Hospital, College of Medicine, Zhejiang University approved using MSC to treating COVID-19. At January 27, 2020, a febrile patient (37.6 °C, working in Wuhan, Hubei Province before arriving to Hangzhou, Zhejiang Province) admitted to our hospital. The patient was positive for COVID-19 at laboratory testing of respiratory secretions obtained by means of endotracheal aspirate, nasopharyngeal swab, or oropharyngeal swab (based on the real-time reverse-transcriptase-polymerase chain reaction (rRT-PCR) amplification of the viral DNA from a sputum sample). Patient with ARDS were defined as those with PaO₂:FiO₂ less than 200 mmHg and bilateral infiltrates consistent with pulmonary oedema on frontal chest radiograph [23]. Laboratory tests of blood, oxygenation indicators, inflammation index, immune indicators, coagulation function and nucleic acid detection of COVID-19 were carried out at Medical Inspection Department of the First Affiliated Hospital at Zhejiang University. The patient was treated with 20 g human immunoglobulin, 1000 mg lopinavir and ritonavir tablets, 600 mg arbidol, and different dose of methylprednisolone in hospital. At the same time, the patient received MSC treatment for three times. High flow oxygen was administered to this patient in intensive care unit (ICU). The patient signed informed consent forms and were willing to share their data.

Biologic measurements

Laboratory tests of blood, oxygenation indicators, inflammation index, immune indicators, coagulation function and nucleic acid detection of COVID-19 were carried out. Other medical information of patients was either retrieved from patients' medical records. Factors which are possible to correlate to clinical features and treatment outcomes in COVID patient with ARDS were analyzed: 1) baseline characteristics including age, underlying conditions, and symptoms; 2) data from laboratory tests and imaging examinations; 3) Combined treatments by basic supporting therapy, antiviral therapy, antibiotic therapy, vasoactive drugs, glucocorticoid therapy, mechanical ventilation, ECMO, ALSS, and CRRT.

Cell transplantation and subsequent observation

The infusion was initiated using a standard blood filter tubing set, and the infusion rate controlled by the investigator based on droplet count. The patient were treated with three fusions of MSCs at the early stage of COVID-19 infection. The injection dose of MSCs is determined as 1 million per kg body weight as previous study [18]. A multiple intravenous infusion of allogeneic MSCs was well tolerated in the patient with COVID-19 induced ARDS.

BLM-induced pulmonary fibrosis and MSC transplantation in mice

To induce pulmonary fibrosis, C57BL/6J mice were anesthetized with 1% pentobarbital sodium (100 mg/ml), then 3U/kg of BLM dissolving in 50 ul phosphate-buffered-saline (PBS) was administered intratracheally, 5×10^5 MSCs in 500 ul PBS were injected into the tail vein of the mice both 24 h and 7 days later after BLM administration, and the group was considered as MSC group; Mice injected with equal PBS was considered as BLM group; Normal C57BL/6J mice served as blank control. After MSCs labeled with luciferase gene, then injected into the mice via tail vein. And a live imaging system was used to observe the migration of MSCs.

Coculture experiments in vitro: MSC/MLE-12 cells and MSC/MLFs

MSCs and MLE-12 cells were seeded on the transwell inserts with a 1:1 ratio. MSCs were seeded in the upper chamber and MLE-12 cells were seeded in the lower chamber. MLE-12 cells stimulated with 5 ng/ml BLM for 24 h in DMEM/F12 medium, then washed with PBS twice before cocultured without MSCs. The three groups are as follows: control group, MLE-12 cells; BLM group, MLE-12 cells treated with BLM; MSC group, MLE-12 cells treated with BLM and cocultured with MSCs.

Similarly, MSCs and MLFs cells were seeded on the transwell inserts with a 1:1 ratio (0.4um poresize; Corning). MSCs were seeded in the upper chamber and MLFs cells were seeded in the lower chamber. MLFs cells stimulated with 3 ng/ml TGF- β 1 (Peprotech, USA) for 48 h without FBS in MEM medium, then washed with PBS twice before cocultured with MSCs for 72 h. The three groups are as follows: control group, MLFs; TGF- β 1 group, MLFs treated with TGF- β 1; MSC group, TGF- β 1 treated with TGF- β 1 and cocultured with MSCs.

Immunohistochemistry and immunofluorescence staining

Washed MLFs/MLE-12 cells with PBS 3 times and fixed with 4% paraformaldehyde for 30 min at room temperature. Next permeabilized with enhanced immunostaining permeabilization buffer for 20 min, and blocked with goat serum for 1 h at room temperature. Then MLFs cells incubated with anti- α -SMA (1:50, Abcam), anti-fibronectin (1:50, Abcam), anti-collagen1 (1:50, Abcam) antibody and MLE-12 cells incubated with E-cadherin (1:250, Abcam), N-cadherin(1:50, Abcam) at 4 °C overnight, followed by Alexa Fluo-488-conjugated goat anti-mouse (1:500, Abcam), and Alexa Fluo-635-conjugated goat anti-rabbit(1:500, Invitrogen) antibodies incubation for 1 h. After washed with PBST 3 times, covered with prolong™ gold antifade mountant with DAPI and coverslips. Finally, images were taken with an Olympus IX-83-FV3000-OSR.

Immunofluorescence analysis was also used to verify whether MenSCs differentiate into injured area cells. Briefly, lung tissues in three groups were embedded in Tissue-Tek O.C.T. compound (Sakura Finetek, Torrance, CA), 5 μ m sections incubated with human surfactant protein D antibody (1:100; santa cruz) at 4 °C overnight, then incubated with Alexa Fluo-635-conjugated goat anti-mouse IgG antibody (1:500; Invitrogen). Next, covered sections with prolong™ gold antifade mountant with DAPI and coverslips. Finally, images were taken with an Olympus IX-83-FV3000-OSR.

RNA sequence and cytokine array

Selected 5 lung tissues samples in three groups, named Control group, BLM group and MSC group, respectively. Then total RNA was extracted using a total RNA kit (QIAGEN) according to the manufacturer's instruction and genomic DNA was removed using DNase I (TaKara). Then RNA quality was determined by 2100 Bioanalyser (Agilent) and quantified using the ND-2000 (NanoDrop Technologies). Only high-quality RNA sample was used to construct sequencing library. After quantified by TBS380, paired-end RNA-seq sequencing library was sequenced with the Illumina HiSeq 4000. The raw data of RNA-seq was in this study have been submitted to the NCBI Gene Expression Omnibus (GEO; <https://www.ncbi.nlm.nih.gov/geo>) under accession number PRJNA514227.

A RayBio® Mouse Cytokine Antibody Array (QAM-CYT-4-8) was used to detected 200 cytokines secreted by MLE-12 cells (n = 3/group). Cell culture supernatants were harvested after culturing 48 h in MLE-12, MLE-12 + BLM, MLE-12 + BLM + MSCs groups. The expression of cytokines was determined by the fluorescent intensities and performed according to the manufacturer's instructions. Other detail information are presented in the supplementary materials and methods.

Data analysis

Baseline data were expressed as mean \pm standard deviation (SD) or median values. To analyze differences between baseline data, Student's t-test analysis was performed on mean numeric data. Statistical analyses were performed using PASW Statistics software version 22.0 from SPSS Inc. (Chicago, IL, USA). P values \leq 0.05 were considered statistically significant.

Results

MSC confirmation and patients characteristics

Flow cytometry was performed to identify the immunophenotyping of menstrual blood-derived MSCs, and the expression of CD29, CD73, and CD105 were positive, while CD34, CD45, CD117, and HLA-DR were negative (**Fig. S1A**). And MSCs can be differentiated into adipogenic, osteogenic, and chondrogenic cells (**Fig. S1B**), which is the same as our previous studies [24].

The first patient had a history of hypertension for several years and worked in Wuhan for a long time. He insisted on oral nifedipine to control blood pressure. Physical examination showed fever, with a body temperature of 39.6 °C, breathing rate of 19 times per minute, the pulse of 110 times per minute. The partial pressure of oxygen (PO₂) is 60.1 mmHg and oxygenation index(PO₂ /FiO₂) is 120.2 on the basis of 50% fraction of inspiration oxygen (FiO₂). The laboratory examination of results showed in **Table S1** with an increased leukocyte count (13.60 * 10⁹/l), decreased lymphocytes (0.5 * 10⁹/l), normal hemoglobin (148 g/l). The inflammatory indicator of and the immune indicator which showed in **Table S1**. The chest computed tomography (CT) indicates large lungs, strands, patches are visible in the two lungs, the border is blurred, part of the paving stones are changed, part of the consolidation, part of the air

bronchus signs (Fig. 1A). The patient tested negative for eight common respiratory pathogens, which were respiratory syncytial virus, adenovirus, influenza A virus, *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, *Legionella pneumophila*, parainfluenza virus, and influenza B virus, and the influenza A antigen screening was also negative. Finally, he was diagnosed with COVID-19 based on the rRT-PCR amplification of the viral DNA from a sputum sample. He was transferred to intensive care unit (ICU) because of severe dyspnea at 28 Jan, 2020. The patient was treated with human immunoglobulin on 27 Jan and 28 Jan. During the ICU from 28 Jan to 05 Feb, methylprednisolone, lopinavir and ritonavir tablets, and arbidol were given to the patient. At the same time, the patient received MSC treatment on 28 Jan, 30 Jan and 01 Feb, 2020. After MSC treatment, the symptoms of fever and dyspnea were significantly improved (Fig. 1B). The PO_2 is 123 mmHg and PO_2/FiO_2 is 332.4 even at 37% FiO_2 on 5 Feb. Hence, the patient was out of the ICU to the general ward at this day and continue methylprednisolone, lopinavir and ritonavir tablets and arbidol treatment. The dynamic changes of blood routine, oxygenation indicators, coagulation function, inflammatory indicators and immune indicators are shown in **Table S2**.

The second patient was found to have little rough breaths. Laboratory examinations showed normal leukocytes ($9.3 \times 10^9/L$), neutrophils ($6 \times 10^9/L$), and lymphocytes ($2.3 \times 10^9/L$). The hypersensitive c-reactive protein (CRP) was 10.8 mg/L. The initial chest CT showed a few interstitial changes in both lungs and ground-glass opacities (GGOs) in the subpleural area of the right lower lobe in right lung. He was given supportive care, and antiviral treatment with lopinavir and ritonavir, and arbidol. Immunoglobulin (20 g daily) was also administrated to modulate the inflammatory response. From day 2 to 7 of hospitalization, the patient continued to have fever, with a daily maximum temperature in the range of 38-39.5°C. On day 5, the patient complained a headache and began to have an obvious coughing, with PaO_2 of 75 mmHg, arterial partial pressure of carbon dioxide ($PaCO_2$) of 33 mmHg, oxygenation index (OI) of 228 mmHg under nasal oxygen breath of 3 L/min. A follow-up chest CT showed an increased density of GGOs in the right lower lobe, which then developed into consolidations with perilobular thickening. Moreover, new patchy shadow appeared in the subpleural area of left lung. Laboratory examination showed almost normal index except increased leukocytes ($10.7 \times 10^9/L$). Apart from symptom management, the patient received methylprednisolone (40 mg daily). On day 7, the development of high fevers reached 39.4°C and the patient got oppression in chest. PaO_2 of 68 mmHg, $PaCO_2$ of 34 mmHg, OI of 208 mmHg under nasal oxygen breath of 3L/min. The following chest CT showing multifocal peripheral patchy areas of nodular consolidations and GGO lesions were newly developed in the subpleural areas of left lung. Due to the patient's recurrent fevers and presentation in chest CT, blood cultures were collected on Day 5, whereas have no growth to date. The patient was permitted interferon alpha inhalation and antibiotic therapies of piperacillin-tazobactam based on the previous comprehensive treatments. Then the physical examination revealed a BT decreased to 38.5°C on Day 11, still accompanied by obvious chest stuffiness and cough with a small amount of sputum expectoration. The Laboratory examination showed sharply increased leukocyte ($25.9 \times 10^9/L$), neutrophils ($23.2 \times 10^9/L$), and CRP was 43.5 mg/L. The inflammation cytokines during this time were also significantly increased. IL-6 was 71.5 pg/ml, IL-10 was 7.8 pg/ml, tumour necrosis factor α (TNF- α) 84.3 pg/ml, tumour necrosis factor γ (TNF- γ) was 34.7 pg/ml. Up to day 11, the patient underwent

persistent positive SARS-CoV-2 RNA at sputum daily. Given the severe pulmonary injury caused by inflammatory response and side effects, the glucocorticoid and antiviral and antibiotic therapies had been withdrawn. The MSCs adoptive transfer therapy was also proposed under the guidance of the specialist group. Treatment with intravenous MSCs therapy was progressed on hospital at Day 11 (29 Jan), 12 (30 Jan), 14 (1 Feb) respectively. During the following three days, the patient felt improved breathe with intermittent dry cough and decreased chest stuffiness. The physical examination revealed improved clinical condition with an obviously decreased BT of 37.4°C. Owing to the changing clinical presentations, methylprednisolone has been given the half dose (20 mg daily). The subsequent chest CT on day 17 revealed significant absorption of worsening patches infiltrating in both lungs. In the meantime, the result of SARS-CoV-2 RNA test at sputum turned negative for the first time, although a repeated rRT-PCR of SARS-CoV-2 RNA was positive on Day 20. Then the persistent negative results of SARS-CoV-2 RNA both at sputum and stool were confirmed in the following days. The temperature of the patient dropped to normal and the symptoms disappeared. On day 23, the chest CT showed almost absorption of leaving a few fibrous lesions maybe represent residual organizing pneumonia. The laboratory results revealed well-improved leukocyte ($14.6 \times 10^9/L$), neutrophils ($10.4 \times 10^9/L$), and levels of inflammation factors. Liver function reflected elevated alanine aminotransferase (ALT = 190 U/L), aspartate aminotransferase (AST = 41 U/L). Given the almost favorable performance of the clinical conditions, on day 24, the patient was discharged.

MSCs improved COVID-19 patients via reducing inflammatory cytokines

We found that after the MSC treatment, the oxygenation indicators (such as $FiO_2\%$, PO_2 , and PO_2/FiO_2) significantly improved, the immune indicators (including CD4, CD8, T lymphocyte, B lymphocyte, and NK cells) were increased and the inflammation indicators (IL-2, IL-6, IL-10, TNF, IFN) were significantly decreased. Repeat chest CT showed stranded and multiple patchy high-density shadows in both lungs, and the exudation lesions in both lungs are significantly better than before (Fig. 1). Additionally, the fibrosis has obviously decreased after MSC transplantation. Finally, the COVID-19 nucleic acid test turned negative at twice. The patient was out of the hospital at 19 Feb without oxygen inhalation and stop methylprednisolone therapy. However, lopinavir and ritonavir tablets and arbidol were continue given to the patient. We follow up the patient at 26 Feb (one week after discharged from hospital) and found that conventional index was normal (Table S1). At last the patient withdrawal all medications. The detailed CONSORT diagram for the treatment of the patient is presented in Fig. 1.

MSCs attenuated inflammation of pulmonary fibrosis in mice

To investigate the effects of MSCs on BLM-induced pulmonary fibrosis, MSCs carried luciferase were transplanted into mice through tail vein twice a week after BLM administration. Live imaging result showed that BLM-treated group recruited more MenSCs migrated into injured lung compared to untreated group (Fig. S2A), while little expression of human specific-surfactant protein D (SPD) revealed that

MenSCs did not differentiate into lung epithelia cells (**Fig. S2B**). According to the results, BLM group displayed less amounts of epithelia cells compared to control group, while this apoptosis was reduced after MSCs administration (Fig. 2A). These results indicated that MSCs migrated to injured lung indicating an anti-apoptosis effect. H&E staining and Masson staining showed that mice from BLM-induced group caused serious damage in the structure of lung alveoli, a large amount of inflammatory cells infiltration and mass deposition of collagen especially around bronchi and vessels compared to control group (Fig. 2B). Although, alveolar structure, inflammatory cells and collagen deposition were decreased when compared to BLM group post MSCs transplantation (Fig. 2B). Additionally, fibrosis area and modified Ashcroft score evaluation were corresponded to the section observation (Fig. 2C and 2D). MSCs transplantation also increased the dry/wet ratio of lung tissues (Fig. 2E). There was significantly increased in collagen deposition in BLM group compared to Control group, while MSCs transplantation showed a decreasing trend of collagen deposition compared to BLM group (Fig. 2F), it was consistent with Masson staining. The body weight of mice in BLM group showed a significant reduction compared to Control group after BLM administration 21 days, and showed a recovery trend after MSCs transplantation (Fig. 2G). These results demonstrated that administration of MSCs improved the symptoms of fibrosis.

According to the cell smears, the number of inflammatory cells were significantly increased in BLM group compared to control group, but it was decreased after MSCs administration (**Fig. S3A**). Total protein of BALF was increased in BLM group but decreased in MSC group (**Fig. S3B and S3C**). Meanwhile, the level of BALF inflammatory cytokines showed same trend. After MSCs administration, the level of IL-10, IL-1 β , IL-6, TGF- β 1 were decreased (**Fig. S3D**). Additionally, serum concentration of IL-10, IL-1 β , IL-6, TGF- β 1 exhibited a same trend as BALF (**Fig. S3E**). These results indicated that MSCs has a potential effect on immune-regulation for reducing lung inflammation.

MSCs reduced BLM-induced apoptosis and EMT of MLE-12 cells

CCK8 assay showed that MSCs significantly reduced BLM-induced MLE-12 cells injury (Fig. 3A) and MSCs promoted the clonogenic potential of MLE-12 cells compared BLM group (Fig. 3B). Additionally, flow cytometry assay confirmed that co-cultured with MSCs significantly reduced the rate of apoptotic MLE-12 cells (Fig. 3C), and MSCs significantly attenuated BLM-induced cell cycle arrest in G2/M phase (Fig. 3D). These results indicated that MSCs had the anti-apoptosis effect on the proliferation of MLE-12 cells after treated with BLM. To assess whether MSCs has an impact on BLM-induced EMT, we investigated the expression of EMT-related proteins. Immunofluorescence assay showed that E-cadherin significantly increased after coculture with MSCs, while N-cadherin significantly decreased (Fig. 3E). Furthermore, wounding healing assay and invasion assay showed that the ability of migration and invasion of BLM-treated MLE-12 cells was significantly inhibited by MSC (Fig. 3F). These results suggested that MSCs has a potential effect in inhibiting EMT in MLE-12 cells.

MSCs relieved BLM-induced pulmonary fibrosis through anti-inflammatory and anti-apoptotic effects

We used an antibody array to examine cytokine levels in the supernatants. PCA analysis showed little differences in the same group, and different groups can be distinguished by a large separation (Fig. 4A). As shown in Fig. 4B, several cytokines (i.e., RANTES, GM-CSF, MIG-1, MCP-5, Eotaxin) were significantly decreased; while OPG, FetuinA, Dkk-1, Renin-1 were highly expressed. GO and KEGG pathway showed that inflammation factors chemotaxis and cytokine-cytokine receptor interaction pathway may be the most important pathway (Fig. 4C and 4D). Furthermore, RNA-Seq was performed on the Illumina HiSeq platform. A total of 2503 genes were found to be significantly differential expressed between Control and BLM group, and 252 significantly differential expressed genes between BLM and MenSC group (Fig. 5A). Among them, 955 genes were found to be up-regulated and 1549 genes down-regulated between Control and BLM group, and 188 genes were found to be up-regulated and 64 genes down-regulated between BLM and MenSC group (Fig. 5B). GO and KEGG analysis showed that apelin signaling pathway, focal adhesion, and NF-kappa B signaling are related to the development of fibrosis (Fig. 5C and 5D). Additionally, α -SMA, CTGF and *cdh1* were related to fibrosis in the apelin signaling pathway (Fig. S4).

As expected, IHC result showed that, after MSCs transplantation, the expression of TGF- β 1, CTGF, α -SMA protein significantly decreased while the expression of E-cadherin was increased compared to BLM group (Fig. 6A). We also measured the expression of upstream protein, results showed that MSC transplantation reduced the expression of phosphorylation of Smad3 (Fig. 6B), although phosphorylation of Smad2 and Smad4 has no significant difference (data not shown). Moreover, Bcl2/Bax/active caspase-3 were considered as the apoptosis-related protein. As shown in Fig. 6B, MSCs treatment resulted in a significant increase of Bcl2 and decrease of BAX and active caspase-3 compared to BLM group. According to these results, we speculated that MSCs has an anti-apoptosis effect to reduce BLM induced pulmonary fibrosis.

Discussion

Patients with COVID-19 virus infections are clinically similar to previous SARS-CoV and MERS-CoV infections [25, 26]. The initial symptoms are usually defined as fever, cough, shortness of breath, with early symptom of sputum, and ARDS, which leads to the final lung injuries and pulmonary fibrosis [27, 28]. This implies that as long as we can solve the breathing difficulties of patients with COVID-19 in a short time, and we guarantee to effectively inhibit the replication of COVID-19. Then treating COVID-19 can wait the patient's own immune system recover by themselves or some immunostimulants, and then successfully resist further virus invasion. In another study of 99 patients, chest pain, insanity, and nausea and vomiting were found in addition to previous findings [29]. X-rays or chest CT images of the patients examined, unilateral or bilateral involvement was found to be compatible with viral pneumonia, and bilateral multilobular and subsegmental consolidation areas were observed in the chest in the intensive care unit [1, 6]. Further autopsy reports found that there was a large amount of sputum in the deceased, which caused severe ARDS, and pathological examination found that there were a good deal of

inflammatory factors in the lung tissue of the patient [12]. Therefore, how to effectively control inflammatory factors, it would be the key to slowing and treating COVID-19 in current situation.

In the process of pulmonary fibrosis, epithelia/endothelia cells were damaged in the earliest stage, then released inflammatory cytokines to initiate a cascade reaction [13]. Early studies have reported that MSCs transplantation may promote lung repair and regulate the process of inflammation to reduce fibrosis [30, 31]. It was first reported that systemically transplanted MSCs has ameliorate fibrotic effects in BLM-induced mice [32]. Many reports have shown that MSCs has a great potential in immunomodulatory effects and attenuates inflammatory responses [33, 34], but the underlying mechanism is still not clear. In our study, we found that late transplantation of MSCs after BLM administration is less effective and more easily to embolize the vein (data not shown). Based on the result above, we choose the day 1 and day 4 for MSCs transplantation after BLM administration in mice. Consistent with other studies [35], early administration of MSCs significantly reduced the damage of lung architecture and inflammatory cells infiltration. Activated/injured epithelia cells release inflammatory mediators to trigger cascade to recruit more leukocytes (neutrophils, T cells, macrophages etc.), which may secrete large number of inflammatory cytokines. TGF- β 1 is widely known as profibrotic and proinflammatory factors participated in the process of pulmonary fibrosis [36]. IL-1 β is regard as a proinflammatory cytokines and increases epithelia damage and alveolar barrier dysfunction [37]. IL-6 is a cytokine with multiple effects in different physiological process of pulmonary fibrosis [38]. IL-10 is generally recognized as an anti-inflammatory cytokine and we speculate that it may be related to the promotion of T cell helper-2 reaction and the increasing of IL-4 and IL-13. In the present study, we found that TGF- β 1, IL-6, IL-1 β in BALF and serum were significantly decreased after MSCs administration, indicated that MSCs shown an anti-fibrotic role through an anti-inflammatory effect, which was coincidence with previous studies in MSC-based therapy for pulmonary fibrosis [30, 39]. Additionally, our study showed that the increasing of IL-10 aggravated BLM-induced pulmonary inflammatory reaction and fibrosis, which was reduced after MSCs administration and it is consistent with previous reports [30]. Interestingly, the levels of RANTES, Eotaxin, GM-CSF, MIP-1 γ , MCP-5 were found to be lower in BLM treated MLE-12 cells after MSC coculture. RANTES and Eotaxin are important chemokines of Eos, which can directly mobilize Eos to migrate into the airway lumen, leading to the enlargement of the inflammatory response [40]. GM-CSF is produced and active at the site of inflammation tissue, and blockade it has a therapeutic effect in different inflammatory disease including cardiac inflammation during aortic aneurysm formation and interstitial lung disease [41]. MIP-1 γ (CCL9) is a chemokine which secreted by FAE and recruit CD11b⁺ dendritic cells, neutralization of MIP-1 γ resulted in a significant reduction of CD11b⁺ DCs in CCR6-deficient mice [42]. MCP-5 is homologous to MCP-1 which recruit neutrophil after bacterial infection and increased in the mouse of pulmonary inflammation [43]. Although these differentiated cytokines are not verified, we believe these cytokines play vital role, and they are deserved to be researched in future. Collectively, MSCs may directly inhibit the development of pulmonary fibrosis through an anti-inflammatory pathway. The detail diagram for this mechanism is shown in Fig. 7, which suggests potential targeting for treating COVID-19 induced ARDS and evolutionary pulmonary fibrosis learned from basic mouse model. Because the artificial liver was used to rescue sever symptom patient at early stage in the first case [44], therefore,

artificial liver combined MSC therapy maybe a better way for treating COVID-19 particularly severe patients.

There is also no evidence for MSC associated long term adverse events in our study. Zheng et al. recently concluded that 12 patients with moderate to severe ARDS developed no infusion toxicities or MSC-related serious adverse events after a single-center, randomized, double-blind, and placebo-controlled trial [45]. Our previous study has also showed that menstrual blood-derived MSC has no obvious side effects upon 5 years' follow-up in four MSC patient [18]. Although the source and dose of MSCs in our study differ from Zheng et al, the consistency in terms of tolerability and safety is encouraging. However, there are no previous reports on the quality of life of COVID-19 patients after hospital discharge. When the patients went back home, they not only lacked activities, but also were isolated by their relatives and neighbors because COVID-19 attack made people fear of infection and death. More seriously, some of cured patients can sometimes reappear the positive of COVID-19 virus, and they have to be isolated once again. This suggests that the quality of life of survivors with COVID-19 infection was lower than that of those without COVID-19. The severity of the diseases may influence the quality of life of the patients.

There are some limitations to this clinical trials. First and foremost, with only 2 patients using MSC, the samples are too small. Thus we cannot generalize our phase one experience. Additionally, because the large proportion of patients are far away from the Hangzhou city (Wuhan city), the transportation of MSC is an issue.

Conclusion

This is the first report using menstrual blood-derived MSC in treating COVID-19 patients. The underlying mechanism is probably that MSCs inhibit epithelia cell apoptosis and reduce the secretion of inflammatory factors to prevent myofibroblasts activity. Along with our previous work [18, 46], MSC have the ability to improve lung function through anti-inflammatory effects both injury lung. Although the clinical research of MSCs are still in its infancy, we believe that MSCs will be a promising tool for future clinical application.

Declarations

Ethics approval and consent to participate

This study was submitted to and approved by the Ethics Committee of the First Affiliated Hospital, Collage of Medicine, Zhejiang University. MSC administration in patient with COVID-19 was conducted in a single center and open-label clinical trial (ChiCTR2000029606).

Consent for publication

Not applicable.

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Authors' contribution

Lanjuan Li, Charlie Xiang and Xiaowei Xu conceived and designed this study; Xin Chen, Liang Yu, Lijun Chen, and Xiaoqin Zheng performed the experiments, collected and analyzed the data, and wrote the manuscript. Lingling Tang, Kaijin Xu, Hongliu Cai, Yu Chen, Shufa Zheng, Zhenyu Xu, Hainv Gao, Jingjing Qu, Yingan Jiang collected and analyzed the data. All authors have read and approved this final manuscript.

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Availability of data and materials

All data analyzed in this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Figures

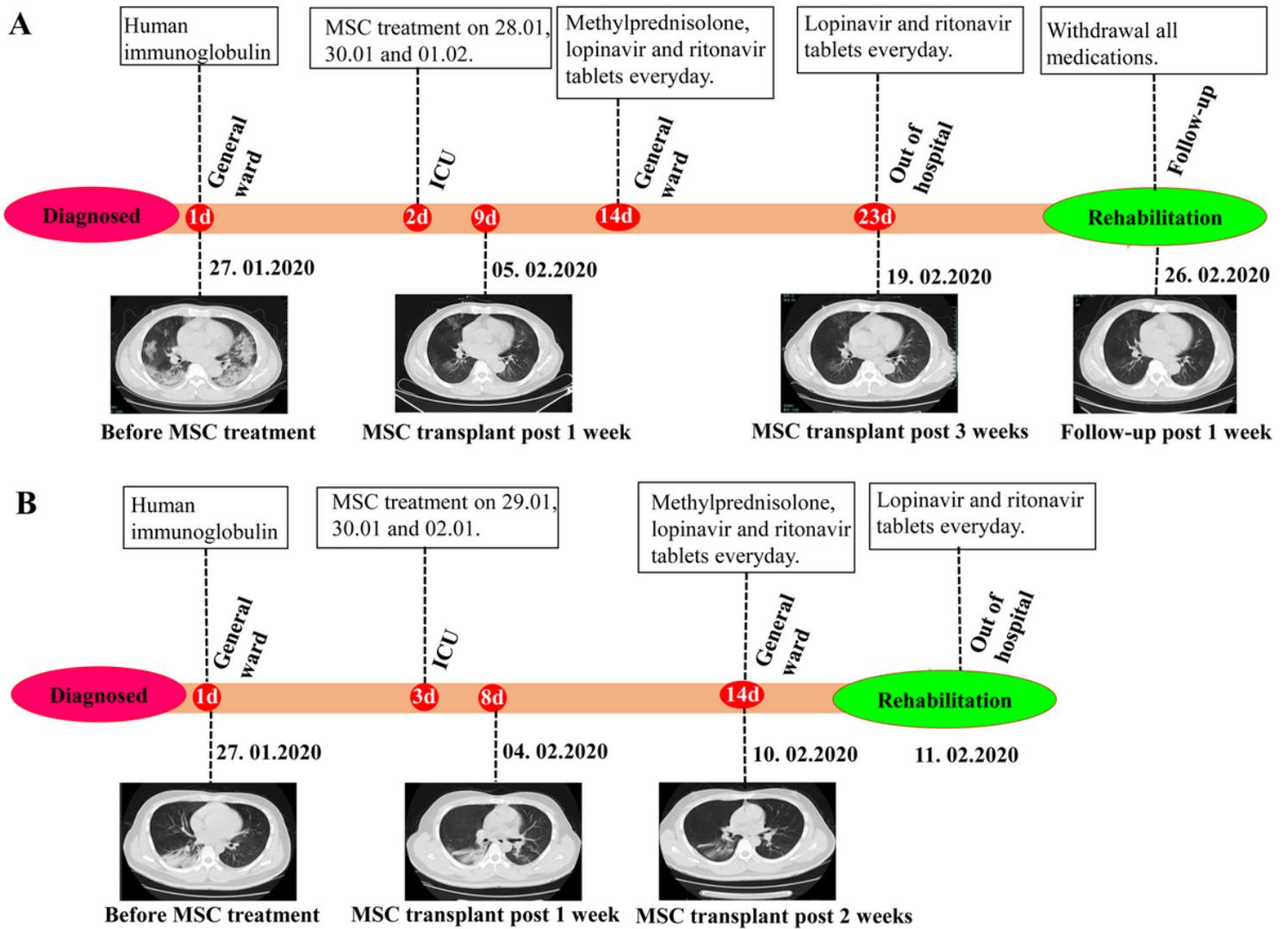


Figure 1

The detailed CONSORT diagram and CT images for the treatment of the two patients are listed in our study. (A) The first patient received MSC treatment for three times with 28 Jan, 30 Jan, and 01 Feb, 2020. (B) And the second patient received MSC treatment for three times with 29 Jan, 30 Jan, and 01 Feb, 2020. After MSC treatment, the symptoms of fever and dyspnea were significantly improved. The oxygenation indicators significantly improved, the immune indicators were increased, and the inflammation indicators were decreased. Repeat chest CT showed stranded and multiple patchy high-density shadows in both lungs, and the exudation lesions in both lungs are significantly better than before. Additionally, the fibrosis has obviously decreased after MSC transplantation. The first patient was out of the hospital at 19 Feb without oxygen inhalation. We follow up the patients at 26 Feb (one week after discharged from hospital) and found that all the laboratory test was normal. The second patient was out of the hospital at 11 Feb without oxygen inhalation.

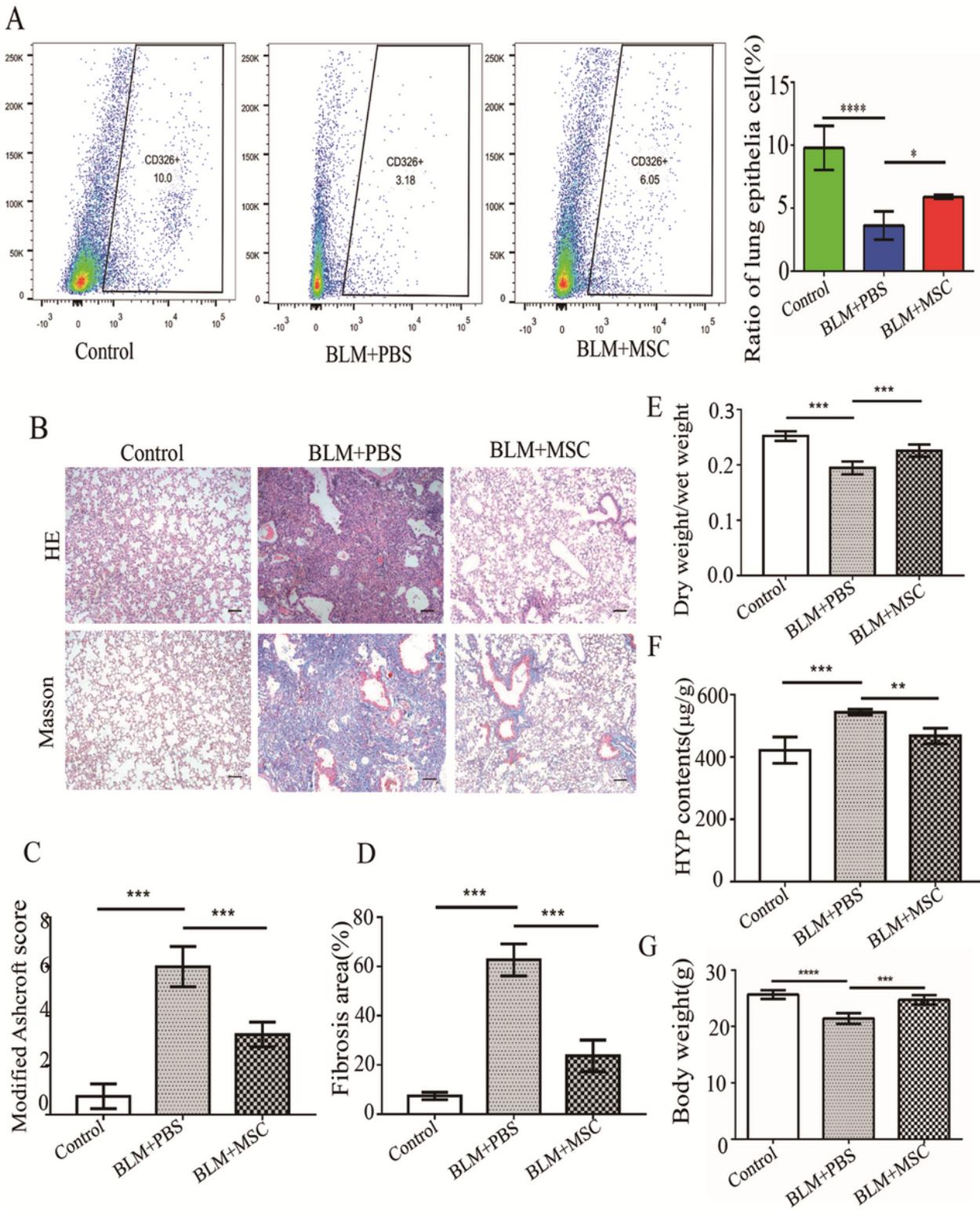


Figure 2

MSCs transplantation reduced epithelia cell apoptosis in vivo and improved fibrosis of lung structure. A, Lung epithelia cell ratio of three different groups. B, H&E staining and Masson staining of lung sections. Severity of pulmonary fibrosis evaluated by modified Ashcroft (C), fibrosis area (D), HYP contents (F). E, dry/wet weight ratio analysis. G, Body weight of mice in three different group. Scale bar: 50µm. *p<0.05, **p<0.01, *** p<0.001.

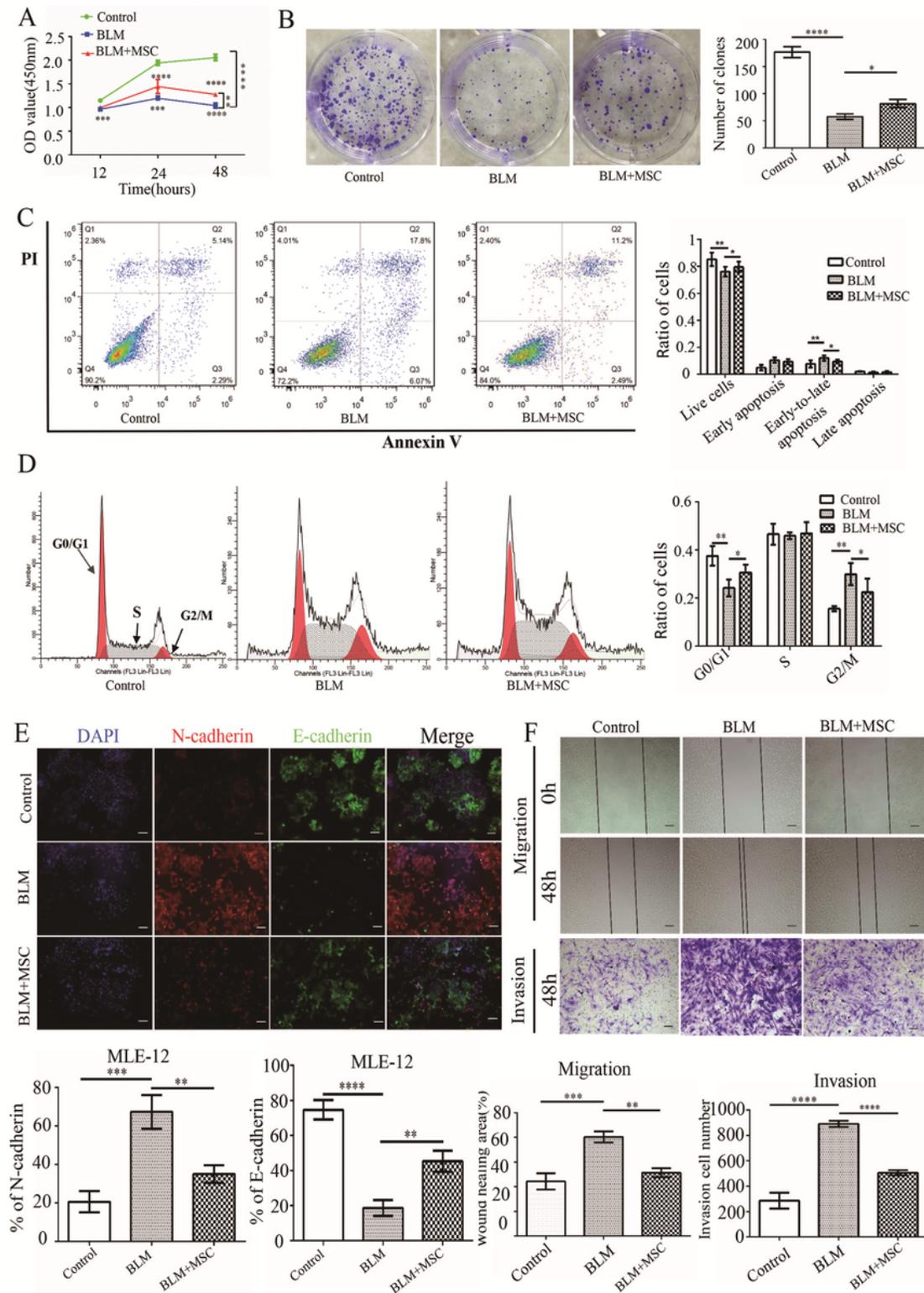


Figure 3

MSCs reduced BLM-induced apoptosis and EMT of MLE-12 cells. A, the anti-apoptosis effect of MSCs on BLM-treated MLE-12 cells examined by CCK8 assay after cocultured 48h. B, clonal formation ability of BLM-treated MLE-12 cells examined by clonogenic assay. Cell apoptosis (C) and cell cycle (D) of BLM-treated MLE-12 cells was performed by flow cytometry. E, the level of E-cadherin and N-cadherin protein of BLM-treated MLE-12 cells were measured by IF after cocultured with MSCs. F, migration and invasion

of BLM-treated/untreated MLE-12 cells were examined by wound healing assay and transwell assay after cocultured with MSCs. Scale bar: 50µm. * $p \leq 0.05$ and ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

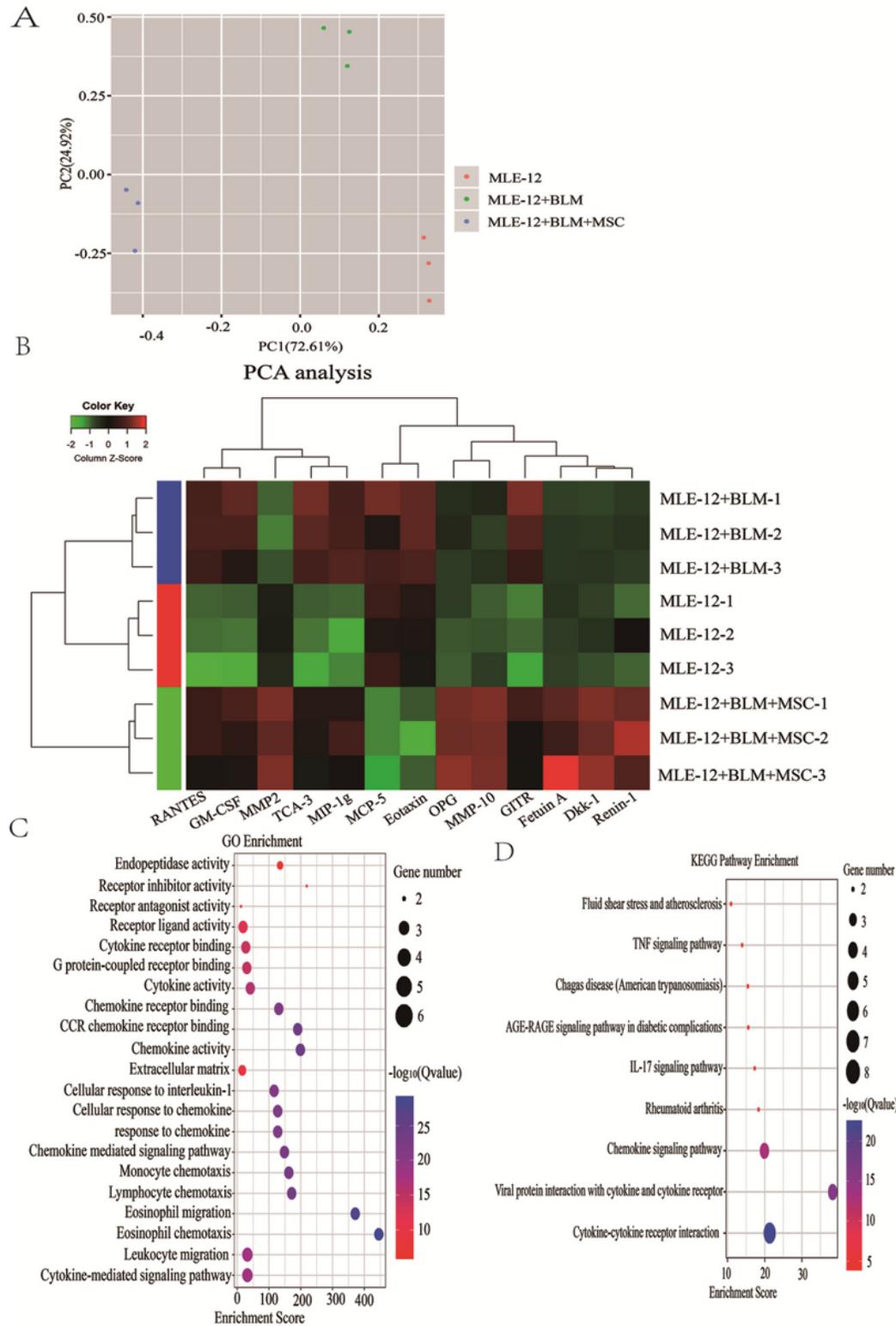


Figure 4

Cytokines detection in BLM-treated MLE-12 cells after MSCs coculture in vitro. A, PCA analysis of three different groups. B, Differential cytokine expression shown as a heat map. Red, green, and black colors

represent upregulated, downregulated, and unchanged expression, respectively. (C and D), significantly different results from GO (C) and KEGG pathway (D) analyses.

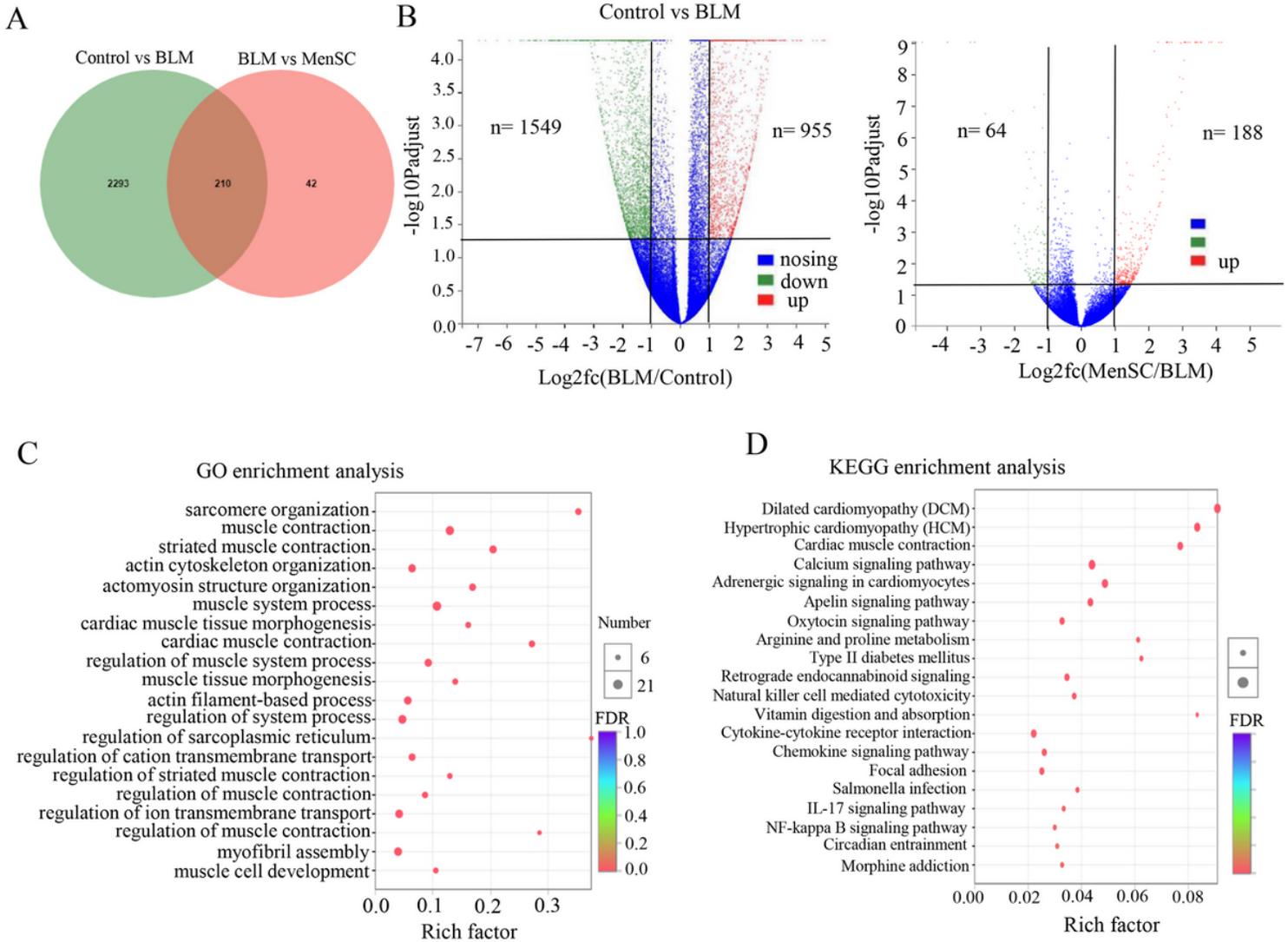


Figure 5

RNA-sequence of lung tissues in three different groups. A, veen analysis between each different two groups. B, volcano showed different expression gene. Red, green, blue color represent upregulated, downregulated, and unchanged expression, respectively. Top 20 significantly different results from GO (C) and KEGG pathway (D) analyses.

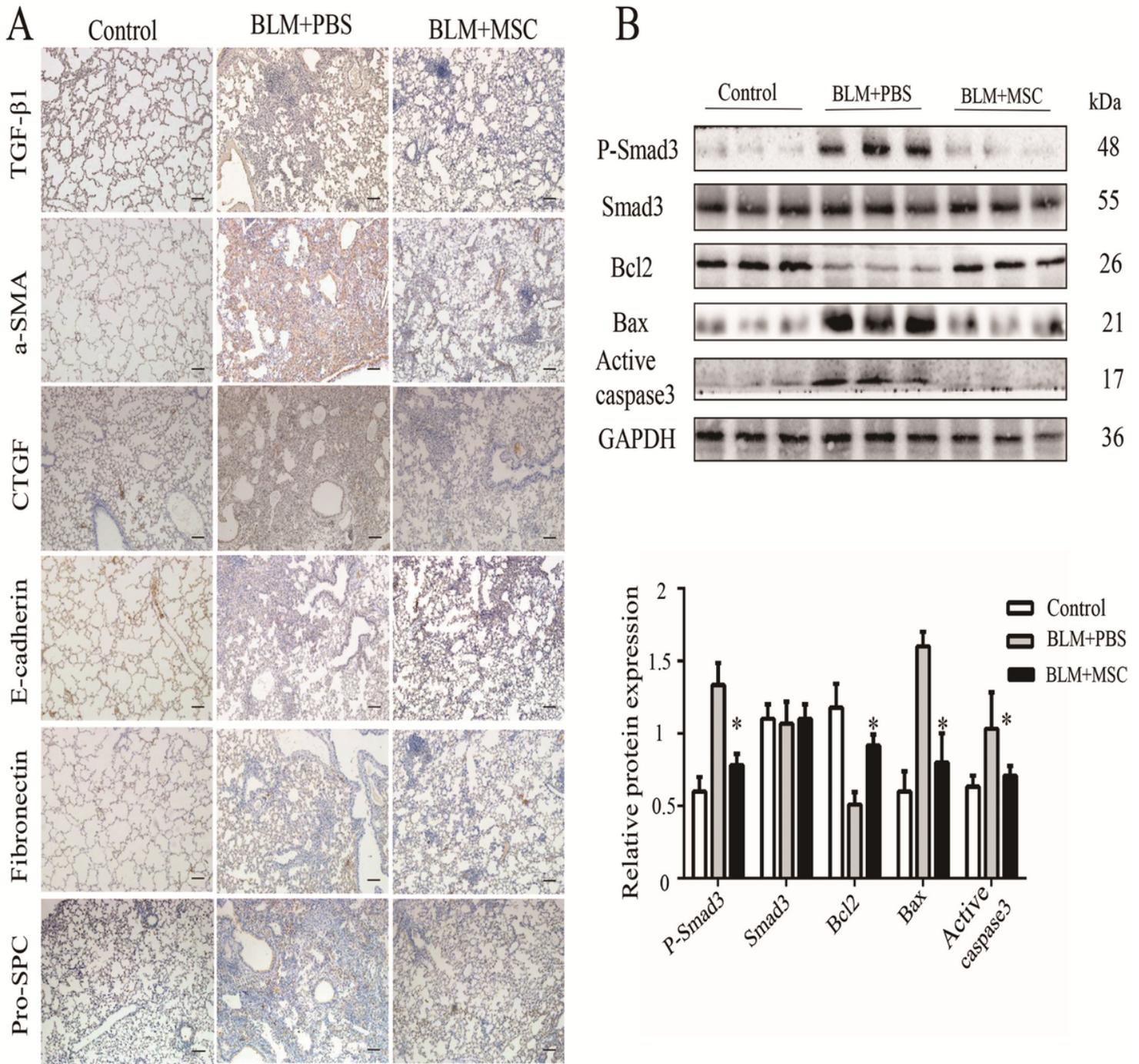


Figure 6

MSCs transplantation promote the repair of lung. A, representative immunohistochemistry images for TGF- β 1, Fibronectin, E-cadherin, CTGF, a-SMA, Pro-SPC. B, western blot analysis protein levels of P-Smad3, Smad3, Active caspase3, Bcl2, Bax, GAPDH in the lung tissues of three different groups. * $p \leq 0.05$. Scale bar: 100 μ m.

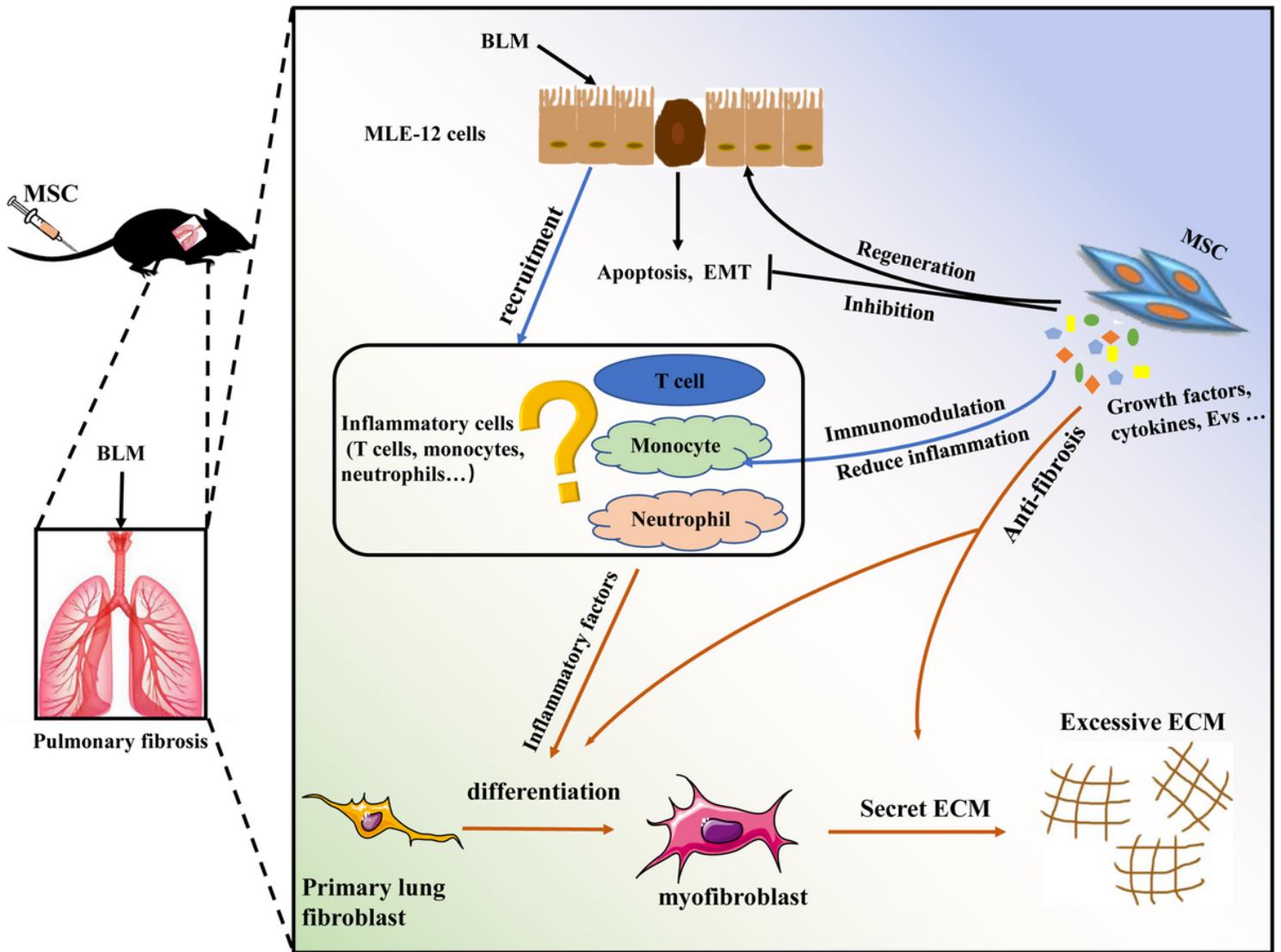


Figure 7

The detail diagram for this mechanism. Potential mechanisms of MSCs in the therapy of pulmonary fibrosis. MSC secrete cytokines to target inflammatory cells to reduce inflammation. It suggests potential targeting sites for pulmonary fibrosis learned from basic mouse model.

Supplementary Files

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